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RESEARCH ARTICLE

Phytochemical Composition, Antioxidant and Antibacterial Activities of *Calophyllum Canum* Hook. f. ex T. Anderson Extracts: Molecular Docking Insights

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ABSTRACT

This study investigates the phytochemical content and biological activities of the *C. canum* stem bark extracts, focusing on its antioxidant and antibacterial potential. The ethyl acetate extract yielded known xanthones of 1-hydroxy-7-methoxyxanthone **(1)**, caloxanthone C **(2)**, trapezifolixanthone **(3)**, ananixanthone **(4)**, euxanthone **(5)**, gentisin **(6)**, 2-hydroxyxanthone **(7)**, *α*-mangostin **(9)**, and a novel compound, canumolactone **(8)**. Sequential maceration using *n*-hexane, dichloromethane, chloroform, ethyl acetate, and methanol was conducted to assess the total phenolic content (TPC) and total flavonoid content (TFC). The ethyl acetate extract had the highest TPC (277.33 mg GAE/g), while the chloroform extract showed the highest TFC (139.56 mg QE/g). The ethyl acetate extract exhibited the strongest antioxidant activity (IC_{50} = 7.52 µg/mL), with a strong negative correlation (r = −0.968) between TPC and DPPH scavenging activity. Antibacterial testing against *Acinetobacter baumannii* revealed significant efficacy, with MIC values as low as 0.056 mg/mL. Molecular docking showed that gentisin had the highest binding energy against Penicillin-binding proteins (PBP). These findings emphasize the importance of solvent selection in bioactive compound extraction and highlight *C. canum* extracts as promising sources of natural antioxidants and antibacterial agents.

1 | Introduction

Calophyllum canum Hook. f. ex T. Anderson, is a large evergreen tree of the Calophyllaceae family, with the taxonomic synonym of *Calophyllum borneense* Vesque, and known by the Malaysian locals as 'bintangor merah'. This species is widely distributed across Malaysia, Sumatra, and Northwestern Borneo, and thrives in well-drained mixed dipterocarp forests to peat swamps, exhibiting its adaptability up to altitudes of 1200 meters [1, 2]. Local communities, particularly in Sarawak, recognize the significance of the plant as a vital source of timber, with its wood serving diverse purposes. Moreover, the latex extracted from *C. canum* is traditionally used in tuba fishing [3]. In addition, the species derives its name from the greyish look of the entire inflorescence, with the epithet 'canum' signifying 'hoary' or 'grey'. Beyond its botanical allure, *C. canum* has captured attention for its chemical composition. Phytochemical studies have revealed the presence of xanthones, triterpenoids, and flavonoids, offering

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potential avenues for pharmaceutical exploration [1, 3–6]. Biological studies further highlight the diverse activities of *C. canum*, including anti-bacterial [7, 8], anti-oxidant [9], cytotoxic [1, 6], and neuroprotective properties [3].

Moreover, antioxidant agents play a pivotal role in protecting the body from oxidative stress by scavenging the free radicals and undergoing the oxidation themselves [10]. Oxidative stress is the imbalance between the oxidants and antioxidants, which is linked with the development and progression of various diseases, including cancer, neurological disorders, metabolic syndrome, cardiovascular diseases, and inflammatory conditions [10, 11]. Moreover, the ability of the plants to scavenge free radicals is often associated with their phenolic and flavonoid content [12]. In this context, the total phenolic and flavonoid content are considered the key measures of the antioxidant strength of the plant extracts. The greater amount of phenolic and flavonoid contents generally indicates a better antioxidant potential in the extracts.

Plant extracts are also increasingly recognized for their antibacterial potential, besides being known for their antioxidant properties. The interest in exploring the antibacterial properties has grown, especially due to the increase in bacteria that are resistant to multiple antibiotics, which urgently needs attention to investigate new and effective antibacterial agents.

Generally found in the environment and soil, *Acinetobacter* species are Gram-negative bacteria that can cause opportunistic infections in hospitalized and immunocompromised people. The infection by *Acinetobacter nosocomialis* has been widely reported [13]. However, *Acinetobacter baumannii* stands out as a highly problematic pathogen due to its remarkable capability to resist multiple antibiotics [14]. This highly adaptable, non-fermenting, and oxygen-requiring bacterium is capable of causing a serious infection, especially in healthcare settings, as it thrives in diverse environments, thus demanding innovative strategies to combat its growing threat [15].

In addition, previous studies have highlighted the antioxidant activity of *C. canum* species, ranging from weak to strong activities in various extracts and different plant parts of this species [7–9]. Moreover, the neuroprotective activity of the compounds isolated from *C. canum*, such as ananixanthone, β-sitosterol, and friedelin, portrayed significant activity in the same order as a known neuroprotectant utilized in the study [3]. Meanwhile, the previous report on the antimicrobial activity of *C. canum* was reported on the ethanol crude using the disc diffusion method, against *S. aureus* and *B. cereus*, showing weak antimicrobial potential [8].

This study examines the total phenolic content (TPC), total flavonoid content (TFC), radical scavenging activity (RSA) using the DPPH method, and the antibacterial properties of *C. canum* extracts against multidrug-resistant (MDR) *A. nosocomialis* Ab06, MDR *A. baumannii* Ab10, and colistin-resistant MDR *A. baumannii* Ab10 Col35. Additionally, the current investigation reports the isolation of compounds from the ethyl acetate extract of *C. canum*. A molecular docking study of all the isolated compounds was conducted using the therapeutic target penicillin-binding protein (PBP) to investigate their binding interactions with the enzyme. This analysis aims to provide insights into the antibacterial mechanism at the molecular level. The findings of this research contribute to the understanding of *C. canum* as a potential source of natural antioxidants and antibacterial agents, paving the way for future therapeutic applications.

2 | Experimental Section

2.1 | General Experimental Procedure

The AR grade solvent (*n*-hexane, dichloromethane, chloroform, ethyl acetate, and methanol) were used for the extraction, isolation, and purification. Gravity column chromatography was performed using silica gel Merck 60 (1.09385.1000), the gel filtration chromatography was performed using sephadex LH-20 (Sigma-Aldrich) as the stationary phase. TLC was conducted on TLC silica gel Merck 60 F254 (1.05554.0001) aluminum sheets. The radial chromatography plate was prepared using silica gel Merck 60 PF254 (1.07749.1000) containing gypsum in 0.5 mm, 1.0 mm, and 2.0 mm thickness. Spots and bands on the TLC and radial chromatography plate were visualized under UV light at 254 nm and 366 nm. 1D and 2D NMR spectral analyses were carried out using Bruker 400 spectrometer which runs at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR) with tetramethylsilane (TMS) as the internal standard.

2.2 | Plant Material

The stem bark of *C. canum* Hook. f. ex T. Anderson was collected in 2021 from the rainforest of Sarawak, Malaysia. The species was identified by a plant taxonomist (Mr. Tinjan anak Kuda) from the Forest Department of Sarawak. The herbarium voucher specimen (UITM 3007) was deposited at the herbarium of Universiti Teknologi MARA, Kota Samarahan, Sarawak. The stem bark was air-dried before being ground with a conventional mill. The stem bark of the species studied possessed a yellowish to light brown outer bark with close-line lenticels. The inner bark was observed to be orange-brown, and the innermost part was reddish brown. The latex from the bark appears clear, yellowish and sticky.

2.3 | Extraction and Isolation of *C. canum* Stem Bark

The ground stem bark (1.0 kg) was extracted with *n*-hexane for 72 h using the cold maceration technique three times. The extract was filtered with Whatman No. 1 filter paper and concentrated under reduced pressure using a BUCHI model R-215 rotary evaporator to yield the crude extract. The crude extract was kept in a dry place until further use. The powdered stem bark was air-dried before being introduced to other solvents. The steps were sequentially repeated with different solvents of increasing polarity: dichloromethane, chloroform, ethyl acetate, and methanol. The ethyl acetate extract underwent a combination of chromatography techniques, including gravity column chromatography, gel filtration chromatography, and radial chromatography, for isolation and purification processes.

The ethyl acetate extract of *C. canum* underwent chromatography on silica gel gravity column chromatography, employing gradient

elution of *n*-hexane to ethyl acetate at varying ratios (100% *n*-hexane to 100% ethyl acetate), producing a total of 21 fractions. Fractions 1 and 2 were combined and subjected to gel filtration chromatography with sephadex LH-20 as the stationary phase, eluted with 100% methanol, resulting in the isolation of 17 fractions labelled from A to Q. Subfractions L-O were further combined and purified with radial chromatography (2.0 mm thickness) using a *n*-hexane to ethyl acetate ratio of 95:5, leading to the isolation of compounds **1** and **2**.

Next, compound fractions 3–4 were chromatographed on a silica gel column using a mobile phase of *n*-hexane to ethyl acetate (100% *n*-hexane to 3:7 *n*-hexane to ethyl acetate ratio), resulting in 8 subfractions labelled from A to H. The subfractions A-B were isolated using sephadex-LH20 with 100% methanol as the eluent, yielding subfractions 1–14. The back subfractions 11–13 were further purified using radial chromatography (1.0 mm thickness) with an *n*-hexane to ethyl acetate ratio of 8:2, leading to the acquisition of compounds **3** and **4**. Subfractions C from fractions 3–4 underwent separation based on molecule size through sephadex LH-20 chromatography using 100% methanol as the eluent, resulting in 12 fractions. Subfractions 4–7 and 8–11 were purified using the same *n*-hexane to ethyl acetate solvent system (8:2 to 1:1) on radial chromatography, yielding compounds **5** and **6,** respectively. Subfraction D was then subjected to gel filtration chromatography with 100% methanol as the eluent, producing 11 subfractions. Subfractions 4–7 were characterized as compound **7**, while subfractions 8–11 were further purified to obtain compounds **8** and **9**. The isolation work of the ethyl acetate extract of *C. canum* is summarized as in Figure S14.

2.4 | Preparation of Stock Solution

The standard stock solution of the *C. canum* extracts was prepared by accurately weighing 10 mg of the extract, which was then dissolved in 10 mL of methanol to afford a concentration of 1000 µg/mL. A similar procedure was followed with gallic acid and quercetin to yield solutions with a concentration of 1000 µg/mL.

2.5 | Antioxidant Assay

The antioxidant activity of the extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [16], with adjustments made according to the number of extracts. The experiment was conducted in triplicate. From the stock solution, 0.2 mL of the extract was diluted in 4.8 mL of methanol to achieve a concentration of 40 µg/mL. A quantity of 19.7 mg of DPPH was dissolved in 1L of methanol to prepare a concentration of 0.05 mM.

One mL of the methanolic extract or standard was added to 3 mL of methanolic DPPH, which was then vigorously shaken. A blank was prepared by utilizing 4 mL of 100% methanol. A control was prepared by mixing 1 mL of methanol to 3 mL of methanolic DPPH, which was promptly measured upon preparation. The absorbance was measured at 517 nm after an hour of incubation at room temperature (25°C). The radical scavenging activity of DPPH was calculated using the following formula:

$$\text{DPPH radical scavenging activity } (\%) = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

2.6 | Total Phenolic Content

The total phenolic content of the extracts was determined using the Folin-Ciocalteu (FC) method [17], with adjustments made according to the number of extracts. The experiment was conducted in triplicate. From the stock solution, 0.3 mL of the extract was diluted in 2.7 mL of methanol to achieve a concentration of 100 µg/mL. For the standard, six concentrations of gallic acid (10–100 µg/mL) were prepared using the serial dilution method. A 10% (v/v) solution of FC reagent was prepared by diluting 6 mL of reagent in 54 mL of distilled water for this experiment. Two grams of Na_2CO_3 were dissolved in 50 mL of distilled water to prepare 4% (w/v) concentration.

A volume of 2.5 mL of FC solution was added to 0.5 mL of methanolic extracts or standards, followed by the addition of 2 mL of Na_2CO_3 solution after one minute. A blank was prepared by mixing 0.5 mL of methanol with 2.5 mL of FC solution and 2 mL of Na_2CO_3 solution. The absorbance was measured at 750 nm after 2-h incubation at room temperature (25°C) and compared with gallic acid equivalents (mg GAE/g extract).

2.7 | Total Flavonoid Content

The total flavonoid content of the extracts was determined using the aluminum chloride calorimetric method [18], with adjustments made according to the number of extracts. The experiment was conducted in triplicate. The methanolic extracts were directly utilized from the stock solution as prepared in *section 3.6*. For the standard, six concentrations of quercetin (10–100 µg/mL) were prepared using the serial dilution method. A 2% (w/v) methanolic solution of $AlCl_3.6H_2O$ was prepared by dissolving 1 g of $AlCl_3.6H_2O$ in 50 mL of methanol for this experiment.

A volume of 2 mL of methanolic $AlCl_3.6H_2O$ was added to 2 mL of methanolic extracts or standards. A blank was prepared by mixing 2 mL of methanol with 2 mL of methanolic $AlCl_3.6H_2O$. The absorbance was measured at 425 nm after an hour incubation at room temperature (25°C) and compared with quercetin equivalents (mg QE/g extract).

2.8 | *A. baumannii* Ab10 Preparation

A. baumannii Ab10 was recovered from 15% glycerol stock in UiTM Cawangan Sarawak Kampus Mukah. The culture was cultured overnight in Mueller-Hinton broth at 37°C. The cultures were subsequently used to prepare fresh, 2-h cultures for MIC.

2.9 | Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) values for each *C. canum* extract were determined using the broth microdilution technique [19, 20] with slight modifications. The medium used in this technique was Mueller-Hilton broth and all plant extracts were dissolved in DMSO before undergoing a twofold serial dilution, to give concentrations of 1.8, 0.9, 0.45, 0.225, 0.113, 0.056, 0.028, and 0.014 mg/mL Fresh, 2-h bacterial cultures were adjusted to an optical density (OD) based on 0.5 McFarland standard (1.5 x 10^8 CFU/mL) and were inoculated in 100 µL each into 96-well microplates along with 100 µL of each concentration of plant extracts. The plates were then incubated at 37.5°C for 18 h to allow the bacteria to grow in the presence of the plant extracts. MIC was determined as the lowest concentration of the plant extract dilutions that inhibited bacterial growth. To determine the MBC, the samples from MIC wells were sub-cultured onto a new agar plate, followed by incubation at 37.5°C for another 18 h. The plant extracts were considered bactericidal if no bacterial growth was observed on the agar plate and bacteriostatic if otherwise.

2.10 | Molecular Docking Studies

The structure of isolated compounds was drawn using Discovery Studio 4.0 (Accelrys, San Diego, USA) and saved in PDB format. Then, the isolated compounds were optimized for geometry and energy by using Avogadro, employing the steepest descent and conjugate gradient method, and using the MMFF94 force field. The crystal structure of Penicillin-binding proteins (PBP) (PDB ID:- 3UDX) with co-crystal inhibitor imipenem was downloaded from the Protein Data Bank (PDB). The hydrogen atoms were added to the protein structure using AutoDockTools. The docking was performed using AutoDock vina [21]. The Grid box for PBP was set for 40 by 40 by 40 with 1.0 Å spacing. The grid box was set to be 1.0 Å, so all the residues were available in an equal-opportunity zone for ligand binding. The docked ligand binding was ranked according to its binding energies. The highest binding affinity was selected, and the binding interactions were visualized using Discovery Studio 4.0 (Accelrys, San Diego, USA). This ligand conformation was carefully inspected, analyzed, and discussed. Redocking the inhibitor imipenem in PBP was done to verify the docking parameters. The redocking of co-crystal inhibitor imipenem compounds was performed using AutoDock Vina using the abovementioned parameters.

2.11 | Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA). Data obtained from the phytochemical analyses and radical scavenging activity assays were pre-processed by checking for normality using the Shapiro–Wilk. No data transformation was required as the assumptions for parametric testing were met, and no outliers were detected. Results are expressed as mean ± standard deviation (SD) of three independent replicates ($n = 3$). Significant differences among the extracts were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test (two-tailed, $\alpha = 0.05$). The IC_{50} values for radical scavenging activity were automatically calculated in GraphPad Prism using non-linear regression analysis. Pearson's correlation coefficient (r) was used to assess the relationship between phytochemical content and antioxidant activity. Differences were considered statistically significant at $p < 0.05$.

TABLE 1 | Extraction yield of *C. canum* extracts.

Crude extracts	Dry yield (g)	Percentage yield (%)
n-Hexane	22.0	2.20
Dichloromethane	15.1	1.51
Chloroform	24.8	2.48
Ethyl acetate	10.9	1.09
Methanol	21.3	2.13

3 | Results and Discussion

3.1 | Extraction and Isolation of *C. canum* Stem Bark

The sequential maceration extraction of the *C. canum* stem bark has yielded oily yellowish-brown *n*-hexane (22.0 g), light brown dichloromethane (15.1 g), dark brown chloroform (24.8 g), reddish-brown ethyl acetate (10.9 g), and reddish-black methanol (21.3 g) crude extracts. The percentage yield of the crude extracts was tabulated in Table 1. The compounds from the hexane and chloroform extracts were previously reported [3]. The isolation work done on the ethyl acetate extract has yielded 1-hydroxy-7-methoxyxanthone **(1)**, caloxanthone C **(2)**, trapezifolixanthone **(3)**, ananixanthone **(4)**, euxanthone **(5)**, gentisin **(6)**, 2-hydroxyxanthone **(7)**, canumolactone **(8)**, and α-mangostin **(9)** (Figure 1). The 1D NMR, IR, UV, and LCMS spectra of compound **8** were displayed in Figures S1–S5.

3.2 | Spectral Data of the Compounds Isolated From the Ethyl Acetate Extract of *C. canum*

Canumolactone (8): *Rf* = 0.76 ($CHCl_3$ 100%); pale orange gum; ^1H NMR (400 MHz, [D_6]acetone): δ = 7.75 (dd,1H, J = 5.8 Hz, 3.3 Hz; H-3), 7.65 (dd, 1H, J = 5.8 Hz, 3.3 Hz; H-4), 4.18–4.28 (m, 2H; H-6), 1.67–1.77 (m, 1H; H-5), 1.40–1.51 (m, 2H; H-3′), 1.36–1.46 (m, 2H; H-2′), 1.32–1.42 (m, 2H; H-5′), 1.30–1.39 (m, 2H; H-6′), 0.94 (t, 3H; H-4′), 0.91 (t, 3H; H-7′); ^{13}C NMR (100 MHz, [D_6]acetone): δ = 168.0 (C-2), 133.5 (C-1′), 132.0 (C-4), 129.6 (C-3), 68.4 (C-6), 39.7 (C-5), 31.2 (C-2′), 29.7 (C-5′), 24.5 (C-3′), 23.6 (C-6′), 14.3 (C-7′), 11.3 (C-4′); IR (film): $\tilde{\nu}$ <3000 (sp3 CH), 1726 (s, C═O), 1465 (C═C), 1265 (C─O), 1465 (═C─H bend) cm^{-1}; UV/vis (methanol): 251, 285 nm; HRMS (HR-LC-ESI, negative): m/z calcd for $C_{12}H_{19}O_3$ $[M-H]^-$: 211.1340; found: 211.1795; purity (>95%) was confirmed by analytical HPLC. This is the first report of the ^1H and ^{13}C NMR spectral data.

Compounds (1–7) and (9): See Supporting Information [3, 22–31].

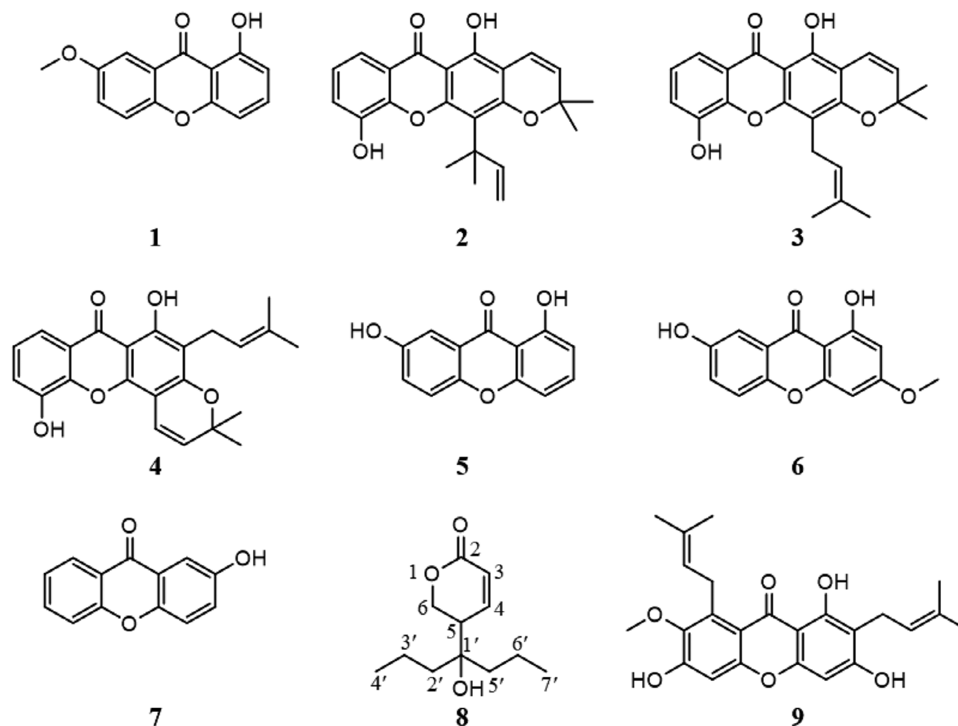

FIGURE 1 | The structure of compounds **1**–**9**.

TABLE 2 | Total phenolic content, total flavonoid content, and DPPH scavenging activity of *C. canum* extracts using different solvents.

Extracts	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	RSA (IC_{50} μg/mL)
CCH	83.07 ± 1.06[a]	11.55 ± 0.19[a]	214.40 ± 41.35[a]
CCD	92.35 ± 1.98[a]	117.96 ± 1.94[b]	156.70 ± 1.86[a]
CCC	96.06 ± 1.52[a]	139.56 ± 1.84[c]	193.40 ± 0.94[a]
CCEA	277.33 ± 5.57[b]	87.66 ± 0.21[d]	7.52 ± 0.07[b]
CCM	223.48 ± 7.44[c]	43.51 ± 1.28[e]	11.45 ± 0.13[b]
Positive control			
Quercetin	—	—	7.48 ± 0.29[b]

CCH: *n*-hexane extract; CCD: dichloromethane extract; CCC: chloroform extract; CCEA: ethyl acetate extract, CCM: methanol extract. The experiment was done in triplicate ($n = 3$) and the data are expressed as mean ± SD. Data within rows with common superscript alphabets are not significantly different from others at TPC ($p < 0.05$), superscript alphabets are not significantly different from others at TFC ($p < 0.05$) and superscript alphabets are not significantly different from others at DPPH ($p < 0.05$) (one-way ANOVA followed by Tukey's test).

3.3 | Total Phenolic Content

The total phenolic content (TPC) of the crude extracts was quantified using the established standard calibration curve of gallic acid ($y = 0.0079x - 0.0773$, $R^2 = 0.9697$). The result obtained revealed significant differences among the extracts, and expressed as milligrams of gallic acid equivalent per gram of dried weight extract (mg GAE/g extract). The TPC was ranged from 83 to 277 mg GAE/g extract as tabulated in Table 2. The ethyl acetate extract contained the highest concentration of phenolic compounds (277.33 mg GAE/g extract), followed by the methanolic extract (223.48 mg GAE/g extract). On the other hand, *n*-hexane, dichloromethane, and chloroform extracts portrayed lower concentration of phenolic compounds, with respective TPC values of 83.07, 92.35, and 96.06 mg GAE/g extract.

3.4 | Total Flavonoid Content

The total flavonoid content (TFC) of the crude extracts was quantified using the established standard calibration curve of quercetin ($y = 0.0321x - 0.0703$, $R^2 = 0.9548$). The result highlighted considerable variability across the extracts, and expressed as milligrams of quercetin equivalent per gram of dried weight extract (mg QE/g extract). The TFC ranging from 11 to 139 mg QE/g extract as tabulated in Table 2. The highest TFC was found in the chloroform extract with the value 139.56 mg QE/g extract, followed by the dichloromethane extract (117.96 mg QE/g extract). Subsequently, the *n*-hexane extract exhibited a flavonoid content of 11.55 mg QE/g extract, while the ethyl acetate and methanolic extracts showed values of 87.66 and 43.51 mg QE/g extract, respectively.

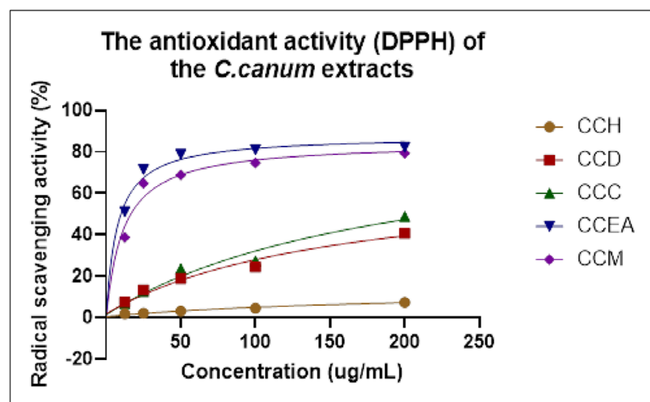

FIGURE 2 | Radical scavenging activity of *C. canum* extracts by DPPH assay. CCH: *n*-hexane extract; CCD: dichloromethane extract; CCC: chloroform extract; CCEA: ethyl acetate extract; CCM: methanol extract.

3.5 | 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity of *C. canum* Extracts

The DPPH radical scavenging activity (RSA) was used to screen the antioxidant potential of the extracts, assessing their ability to neutralize free radicals. A lower IC_{50} indicates a better antioxidant activity. The antioxidant potential is categorized according to the IC_{50} values: <10 µg/mL: very strongly active, 10–50 µg/mL: strongly active, 51–100 µg/mL: moderately active, 101–250 µg/mL: weakly active, >250 µg/mL: inactive [12]. The activity shown by the *C. canum* extracts is noted to be varied across the extracts, with IC_{50} ranging from 7.52 to 214.4 µg/mL (Table 2). The strongest radical scavenging activity was shown by the ethyl acetate extract with IC_{50} 7.52 µg/mL, followed closely by the methanol extract (IC_{50} 11.45 µg/mL), which is comparable with the standard quercetin. The dichloromethane extract gave a weak activity with IC_{50} 156.70 µg/mL. Meanwhile, the *n*-hexane and chloroform extracts portrayed a comparably weaker scavenging activity with respective IC_{50} 214.40 and 193.40 µg/mL. The result was tabulated in Table 2, and Figure 2 depicts the radical scavenging activity (%) against concentration.

3.6 | Pearson's Correlation of the Phytochemical Content and Antioxidant Activity of the *C. canum* Extracts

The positive coefficient (r) shows a direct relationship where both variables progress in the same direction and a strong positive correlation (close to +1) signify a robust relationship. Meanwhile, a strong negative coefficient implies an inverse relationship, where one variable decreases as the other tends to increase, particularly when the coefficient r is close to −1. A significant negative correlation is observed between TPC and RSA with coefficient $r = -0.968$ ($p = 0.007$). The strong negative correlation indicates that the higher the TPC value, the lower the IC_{50} value which indicates the high amount of TPC is associated to a better antioxidant activity. In opposite, the correlation between the TFC and RSA was statistically not significant (p = 0.783) and weak (r = 0.171) suggesting no meaningful relationship between TFC and RSA. Moreover, the correlation between TPC and TFC was also non-significantly weak ($r = -0.134$, $p = 0.830$), implying the amount of TPC does not affect the amount of TFC. Based on this analysis in Table 3, the correlation between TPC and RSA has met the criterion, spotlighting the role of phenolic compounds in improving the antioxidant activity.

3.7 | Antibacterial Activity of *C. canum* Extracts

The antibacterial activity of five extracts from *C. canum* was evaluated against multidrug-resistant (MDR) *A. nosocomialis* Ab06, MDR *A. baumannii* Ab10, and colistin-resistant MDR *A. baumannii* Ab10 Col35. The extracts tested included *n*-hexane (CCH), dichloromethane (CCD), chloroform (CCC), ethyl acetate (CCEA), and methanol (CCM). In this study, DMSO was used as the negative control while erythromycin was used as the positive control due to the bacterial strains being erythromycin-susceptible isolates. The MIC and MBC results are presented in Table 4. A positive sign (+) indicates bacterial growth while a negative sign (−) indicates no growth was observed for MBC determination. All the results obtained for MIC and MBC determination are provided in Figures S6–S13.

The plant extracts with a MIC value lower than 0.100 mg/mL is considered highly active while MIC values ranging from 0.100–0.625 mg/mL indicate moderate activity [32]. By following the threshold, in this study, the ethyl acetate extract exhibited the most significant antibacterial activity against Ab06, Ab10 Col35, and Ab10, with MIC values of 0.450, 0.113, and 0.056 mg/mL, respectively. Other extracts including chloroform (CCC) and methanol (CCM) extracts, displayed moderate activity across all tested strains, with MIC values consistently at 0.45 mg/mL for Ab06 and lower for Ab10 and Ab10 Col35. In contrast, the *n*-hexane (CCH) extract of *C. canum* was the least active, with no inhibitory effect on Ab06 and minimal activity on Ab10 Col35 and Ab10 at a concentration of 0.225 mg/mL.

3.8 | Molecular Docking Studies

A previous study reported that *β*-lactam antibiotic disrupts cell-wall synthesis in gram-negative bacteria by covalently inactivating enzymes known as penicillin-binding protein (PBP). The primary function of PBP is to help the gram-negative bacteria's cells, which are protective layers around the inner layer that help maintain the structural integrity of the cell [33]. When PBPs are inhibited, the cell wall cannot maintain its structure. This weakness causes the cell wall to rupture, ultimately killing the gram-negative bacteria. Therefore, the inhibition of PBP is considered a practical approach to treating gram-negative bacteria [34]. Redocking of inhibitor, namely, imipenem, to the crystal structure of PBP was performed to verify the docking parameter used in this study. This was done to ensure the reproducibility of the docking outputs. The orientation of the best docking pose of the inhibitor was superimposed with the inhibitor's original crystal structure at the crystal structure's binding side [35]. The root means square derivative (RMSD) value between top-ranked docked imipenem conformation with crystal pose of imipenem was found to be 1.6691 Å. The molecular docking results show that xanthone named gentisin shows the highest binding energy compared to another compound (Figure 3). Meanwhile, the new compound,

TABLE 3 | The correlation between TPC, TFC, and DPPH radical scavenging activity (RSA).

	TPC		TFC		RSA	
	Coefficient (r)	*p*-Value	Coefficient (r)	*p*-Value	Coefficient (r)	*p*-Value
TPC	1.000	—	−0.134	0.830	−0.968	0.007
TFC	−0.134	0.830	1.000	—	0.171	0.783
RSA	−0.968	0.007	0.171	0.783	1.000	—

TABLE 4 | MIC and MBC values (mg/mL) for *C. canum* extracts against three *A. baumannii* bacterial strains.

Extracts	Ab06		Ab10 Col35		Ab10	
	MIC	MBC	MIC	MBC	MIC	MBC
CCH	ND	ND	0.225	+	0.225	+
CCD	ND	ND	0.225	+	0.113	+
CCC	0.450	+	0.113	+	0.113	+
CCEA	0.450	+	0.113	+	0.056	+
CCM	0.450	+	0.225	+	0.225	+

ND = not determined.

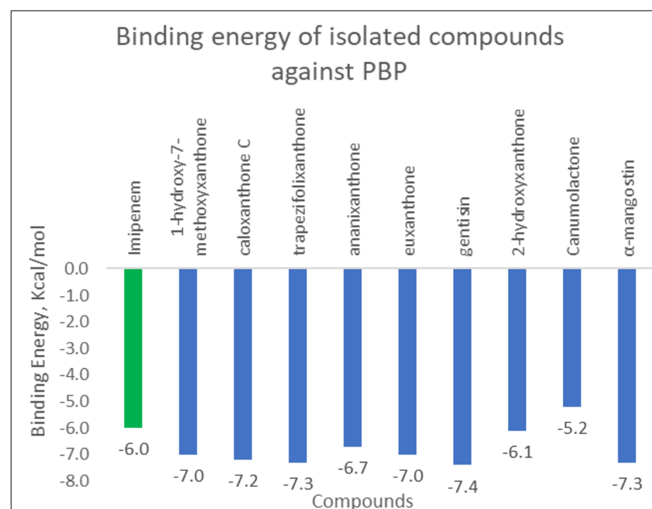

FIGURE 3 | Binding energy of isolated compounds from *C. canum* against PBP.

canumolactone shows the lowest binding energy indicating weak antibacterial activities. The high binding energy of gentisin can be attributed to the formation of 7 hydrogen bonds between gentisin and the amino acid residues of penicillin-binding protein (PBP), along with additional binding interactions such as van der Waals forces and carbon–hydrogen bonds (Figure 4). The hydrogen bonds are contributed by methoxy, hydroxy and carbonyl groups attached to the xanthone skeleton. Furthermore, gentisin was found to interact with specific amino acid residues, including ASN434, THR670, THR672, and TYR707, which are also involved in the binding mechanism of β-lactam antibiotics [34]. This similarity suggests that gentisin may exhibit a comparable mode of action to β-lactam antibiotics, potentially influencing its antimicrobial efficacy.

3.9 | Discussion

Xanthones are commonly found in plants of the genus *Calophyllum*, and known for their diverse biological activities [36]. The current study focused on isolating compounds from the ethyl acetate extract of *C. canum*, resulting in the identification of known xanthones, 1-hydroxy-7-methoxyxanthone **(1)**, caloxanthone C **(2)**, trapezifolixanthone **(3)**, ananixanthone **(4)**, euxanthone **(5)**, gentisin **(6)**, 2-hydroxyxanthone **(7)**, α-mangostin **(9)**, and a new six-membered ring lactone, with a trivial name canumolactone **(8)** (Figure 1). Compound **8** was isolated as a pale orange gum with IUPAC name 5-(4-hydroxyheptan-4-yl)-5,6-dihydro-2*H*-pyran-2-one. The LCMS spectrum (Figure S3) showed a molecular peak at *m/z* 212.3 which is in line with the molecular formula $C_{12}H_{20}O_3$. The IR spectrum (Figure S4) exhibited sp^3 hybridized —CH (CH, CH_2, CH_3) stretch at <3000 cm^{-1}, conjugated C═O peak in ester at 1726 cm^{-1}, and C—O stretches (1226, 1122, and 1071 cm^{-1}). The UV spectrum (Figure S5) displayed the absorptions at 250 and 280 nm indicating the presence of conjugation system in the compound.

Based on the result, the TPC increased when the solvent polarity was increased. Moreover, ethyl acetate and methanol are the most effective solvents for the extraction of phenolic compounds especially in the sequential maceration extraction. The extraction technique employed extracted the nonpolar and less polar compounds in the earlier stage, leaving semi- and polar compounds at the end of extraction. Previous literature [8] has reported a similar pattern whereby TPC values increased along with the solvent polarity, ranging from 1–3 μg Gallic acid/10 mg extract. On the other hand, the amount of TFC in the semi-polar solvents particularly, dichloromethane and chloroform extracts were the highest. This suggests that semi-polar solvent is the most efficacious in extracting the flavonoid compounds when applying the sequential extraction maceration technique. Meanwhile, lower TFC was observed in ethyl acetate and methanol extracts indicating a lower amount of polar flavonoid compounds. On the side note, previous report [8] has indicated a different pattern of TFC where the content of the total flavonoid increase as the more polar solvent was used.

Flavonoids are sub-group of phenolic compounds with ability to scavenge a wide range of oxygen, chlorine and nitrogen species such as hydroxyl ions, peroxynitrous acid, superoxide, reactive oxygen, peroxylradicals, and hypochlorious acid to their ability to

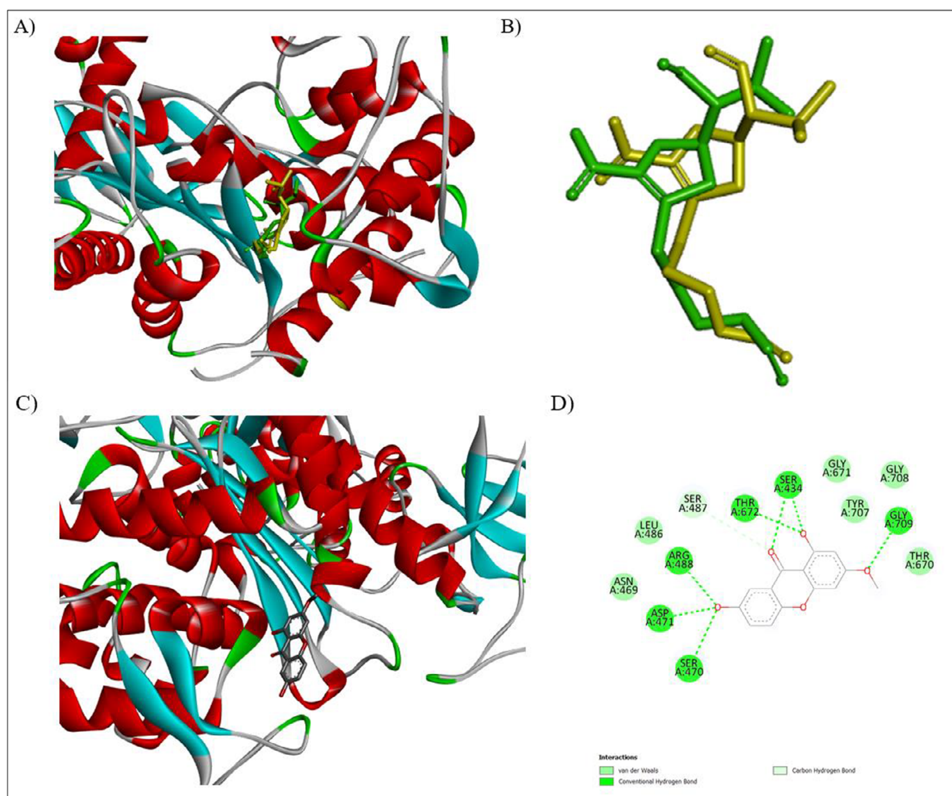

FIGURE 4 | Diagrams showing (A) the alignment of the redocked imipenem (green stick model) and imipenem co-crystallized (yellow stick model) within the binding site of PBP; (B) superimposition of redocking imipenem (green stick model) and imipenem crystal (yellow stick model); (C) gentisin within the binding site of PBP; (D) the 2D schematic diagram of residues in the binding site of PBP exhibiting hydrogen bond and hydrophobic interaction with gentisin visualized and analyze by using Discovery studio 4.0.

chelate ions by decreasing the metal ions pro-oxidant capacity [37, 38]. Moreover, phenolic, and flavonoid compounds are often associated with the antioxidant potential of the plants [38]. Hence, the correlation of TPC, TFC, and RSA was analyzed using the Pearson's correlation. The Pearson's correlation analysis indicates no correlation between TPC and TFC. In other word, TFC is not affected by the TPC. Meanwhile, TPC and RSA gave a strong negative correlation based on the analysis, which stipulates the TPC affect the antioxidant potential inversely. Figure 5 shows the high quantity of phenolic compounds in the ethyl acetate and methanol extracts has resulted in low IC_{50} values, demonstrating a potent antioxidant activity.

The strongest antioxidant potential was demonstrated by ethyl acetate extract, with IC_{50} 7.52, followed by methanolic extract (IC_{50} 11.45), which are comparable with the standard, quercetin (IC_{50} 7.48). The significant difference in the radical scavenging activity (IC_{50}) observed between the less polar extracts (CCH, CCD, and CCC) and more polar extracts (CCEA and CCM) spotlighting the role of solvent in the extraction of phenolic compounds, especially for the extraction technique applied. This technique has resulted in the extraction of compounds of similar polarity with the solvent used. As a result, high concentration of polar phenolic compounds is found in ethyl acetate and methanol extracts, which contributes to a more potent antioxidant activity. In addition, the previous study [8] has reported a similar trend where TPC values increase, the antioxidant activity increase.

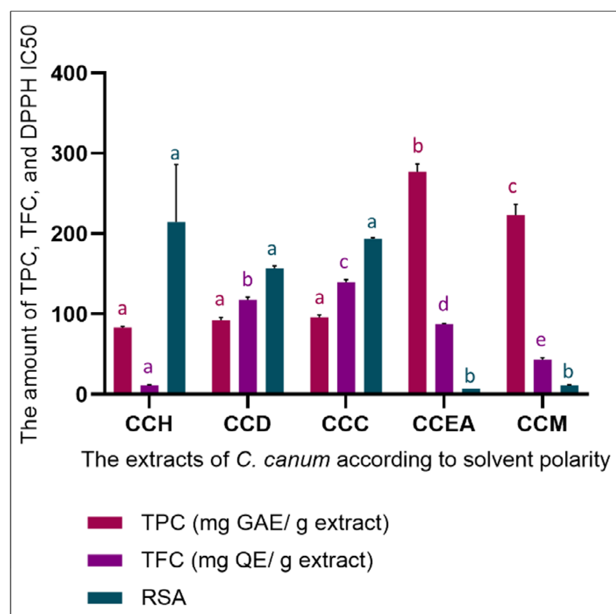

FIGURE 5 | The bar chart of TPC, TFC, and RSA of the *C. canum* extracts. CCH: n-hexane extract; CCD: dichloromethane extract; CCC: chloroform extract; CCEA: ethyl acetate extract, CCM: methanol extract. The experiment was done in triplicate ($n = 3$) and the data are expressed as mean $\pm$ SD. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test. Bars with different superscript letters indicate significant differences among extracts at $p < 0.05$.

For the antibacterial assay, the ethyl acetate (CCEA) extract of *C. canum* demonstrated the most significant antibacterial activity among all the extracts, particularly against Ab10 as shown by its lowest MIC values at 0.056 mg/mL. This result is consistent with findings from previous studies, where methanol and ethyl acetate extracts of *Calophyllum* species exhibited strong biological activity such as antioxidant capacity [39], possibly due to the solvent's ability to isolate more phytochemical compounds efficiently [40, 41]. The results suggest that the bioactive components in the ethyl acetate extract could possess broad-spectrum activity. A higher concentration of more than 1.8 mg/mL for all extracts was not tested in this study as an extract is considered inactive when its MIC value is above 1.000 mg/mL [42]. However, these findings are contradictory from the previous study [8]. In the study, the *n*-hexane and dichloromethane extract of the *C. canum* exhibited more significant activity than the methanol extract.

Although all three bacterial strains belong to the same genus, the inhibitory effects of extracts on them were different as Ab06 required the highest extract concentrations for inhibition with MIC value at 0.450 mg/mL for chloroform, ethyl acetate, and methanol extracts. This could be due to its multidrug-resistant profile. This observation aligns with studies indicating that environmental factors, such as the bacterial strain's origin and microbial exposure, may influence its resistance, thus requiring higher inhibitory concentrations [43]. For instance, Ab06 is described as a multidrug-resistant isolate its resistance likely comes from adaptation in an environment that was exposed to antibiotics, making it harder to inhibit. From the MBC results, it was determined that most of the *C. canum* extracts are bacteriostatic as bacterial growth was observed after the incubation of samples sub-cultured from the MIC findings, except for *n*-hexane and dichloromethane extracts.

4 | Conclusion

The isolation of the ethyl acetate extract of *C. canum* has yielded 8 known xanthones and a new six membered cyclic lactone. The ethyl acetate is the best solvent for the phenolic compounds extraction while chloroform is the efficacious solvent in extracting flavonoid compounds when the sequential maceration extraction technique was employed. Other than that, the presence of the phenolic compounds in the extracts directly contribute to the antioxidant potential as well as exhibited the most significant antibacterial activity against *A. baumannii* strain, particularly Ab10, with the lowest MIC values. Meanwhile, the *n*-hexane extract showed the least activity, indicating the choice of solvent extraction affects the bioactive compounds extracted from the plant sample. Molecular docking studies show that gentisin has the highest binding energy of all isolated compounds. The attachment of the substituents, such as methoxy and hydroxy groups contributes the activity.

Author Contributions

Mas Atikah Lizazman: writing – original draft and preparation resources. **Vivien Jong Yi Mian:** conceptualization, review, supervision, and funding acquisition. **Thiruventhan Karunakaran:** review and supervision. **Nor Hisam Zamakshshari:** writing – original draft, review, and supervision. **Yiizamy Suffian:** writing – original draft. **Asla Marleena Nazeri1, Nik Zakuan Hakim Bin Nik Mohd Nazri, Mohamad Izwan Bin Ismail, and Kirnpal Kaur Banga Singh:** data acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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Supporting Information

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